

## Studies of the CobA-Type ATP:Co(I)rrinoid Adenosyltransferase Enzyme of *Methanosarcina mazei* Strain Gö1

Nicole R. Buan,<sup>†</sup> Kimberly Rehfeld, and Jorge C. Escalante-Semerena\*

Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin 53726

Received 17 January 2006/Accepted 28 February 2006

Although methanogenic archaea use B<sub>12</sub> extensively as a methyl carrier for methanogenesis, little is known about B<sub>12</sub> metabolism in these prokaryotes or any other archaea. To improve our understanding of how B<sub>12</sub> metabolism differs between bacteria and archaea, the gene encoding the ATP:co(I)rrinoid adenosyltransferase in *Methanosarcina mazei* strain Gö1 (open reading frame MM3138, referred to as *cobA<sub>Mm</sub>* here) was cloned and used to restore coenzyme B<sub>12</sub> synthesis in a *Salmonella enterica* strain lacking the housekeeping CobA enzyme. *cobA<sub>Mm</sub>* protein was purified and its initial biochemical analysis performed. In vitro, the activity is enhanced 2.5-fold by the addition of Ca<sup>2+</sup> ions, but the activity was not enhanced by Mg<sup>2+</sup> and, unlike the *S. enterica* CobA enzyme, it was >50% inhibited by Mn<sup>2+</sup>. The CobA<sub>Mm</sub> enzyme had a  $K_m^{\text{ATP}}$  of 3  $\mu\text{M}$  and a  $K_m^{\text{HOCbl}}$  of 1  $\mu\text{M}$ . Unlike the *S. enterica* enzyme, CobA<sub>Mm</sub> used cobalamin (Cbl) as a substrate better than cobinamide (Cbi; a Cbl precursor); the  $\beta$  phosphate of ATP was required for binding to the enzyme. A striking difference between CobA<sub>Se</sub> and CobA<sub>Mm</sub> was the use of ADP as a substrate by CobA<sub>Mm</sub>, suggesting an important role for the  $\gamma$  phosphate of ATP in binding. The results from <sup>31</sup>P-nuclear magnetic resonance spectroscopy experiments showed that triphosphate (PPP<sub>i</sub>) is the reaction by-product; no cleavage of PPP<sub>i</sub> was observed, and the enzyme was only slightly inhibited by pyrophosphate (PP<sub>i</sub>). The data suggested substantial variations in ATP binding and probably corrinoid binding between CobA<sub>Se</sub> and CobA<sub>Mm</sub> enzymes.

Bioinformatics analyses of archaeal genome sequences readily identify putative orthologs of B<sub>12</sub> biosynthetic genes found in *Salmonella enterica* (28, 44). However, positive identification of gene function requires in vivo and in vitro analyses. To facilitate the latter, we have taken advantage of our extensive collection of *S. enterica* strains lacking specific functions needed for coenzyme B<sub>12</sub> synthesis. We have successfully used this approach to identify archaeal nonorthologous replacements of bacterial B<sub>12</sub> biosynthetic functions needed for the assembly of the nucleotide loop of coenzyme B<sub>12</sub> (25, 44, 47, 48, 50, 51) and for the transport of corrinoids across the membrane (49).

In these studies we turned our attention to the archaeal enzyme responsible for the conversion of vitamin B<sub>12</sub> to its coenzymic form, adenosyl-B<sub>12</sub> (AdoB<sub>12</sub>, AdoCbl). Not all archaeal genomes contain orthologs of the *S. enterica* *cobA* gene, which in this bacterium and in *Escherichia coli* encodes the housekeeping enzyme responsible for attaching the adenosyl moiety from ATP to the cobalt ion of the corrin ring (13, 24, 41). In *S. enterica*, the *cobA* gene is constitutively expressed, and the activity of the CobA<sub>Se</sub> enzyme is required for de novo biosynthesis of the corrin ring (13) and for salvaging complete and incomplete corrinoids from the environment (39–41). In other words, the broad specificity of the CobA<sub>Se</sub> enzyme allows *S. enterica* to salvage a broad spectrum of corrinoids from its environment and to solve the need for corrinoid adenosylation during de novo corrin ring biosynthesis.

The genome of the methanogenic archaeon *Methanosarcina mazei* strain Gö1 contains a putative *cobA* ortholog (ORF

MM3138), which we hypothesized might play physiological roles similar to those of the CobA<sub>Se</sub> enzyme.

### MATERIALS AND METHODS

**Plasmid construction.** The *M. mazei* *cobA*<sup>+</sup> gene (locus tag MM3138) was PCR amplified from *Methanosarcina mazei* strain Gö1 genomic DNA by using the primers “M.mazei CobA pT7-7 fwd” (5′-TACGGGCCATATGCGCGGTGCATGGTATAC-3′) and “M.mazei CobA pT7-7 rev” (5′-GGATCCGTGATCAATATTCAGTCCTTCCCTTGCAG-3′). The PCR fragment was digested with NdeI/BamHI and ligated into cloning vector pT7-7 (42), yielding plasmid pMmaCOBA4.

**Analysis of growth behavior.** Strains and plasmids used in the present study are listed in Table 1. The ability of the *M. mazei* *cobA*<sup>+</sup> gene to complement a *Salmonella* *cobA* strain was assessed in 96-well plates (Becton/Dickinson). Strains were grown in lysogenic broth (LB) (4) (2 ml) overnight cultures containing the appropriate drug at 37°C. Plates contained no-carbon E (NCE) minimal medium (3), trace minerals (1), MgSO<sub>4</sub> (1 mM), and NH<sub>4</sub>Cl (30 mM). When cells were grown on glycerol as the sole carbon and energy source, the medium was supplemented with glycerol (30 mM), 5,6-dimethylbenzimidazole in dimethyl sulfoxide (300  $\mu\text{M}$ ), and dicyanocobinamide [(CN)<sub>2</sub>Cbi, 200 nM]. When cells were grown on ethanolamine, the medium was supplemented with ethanolamine hydrochloride (30 mM, pH 7), methionine (0.5 mM), and hydroxocobalamin (HOCbl, 200 nM). Samples (10  $\mu\text{l}$ ) of overnight LB cultures containing the appropriate drug were used to inoculate each well containing 190  $\mu\text{l}$  of fresh medium. Plates were incubated in a BioTek plate reader at 37°C with maximum aeration.

**Isolation of the CobA<sub>Mm</sub> protein.** Plasmid pMmaCOBA4 was transformed into *E. coli* BL21(ΔDE3) for overexpression (17). Fresh transformants were grown overnight in 10 ml of LB plus ampicillin (100  $\mu\text{g}/\text{ml}$ ). The latter cultures were used to inoculate three 1.5-liter flasks containing LB plus ampicillin. Cells were shaken at 37°C until late log phase, when gene overexpression was induced with 0.5 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside), and the flasks were shifted to 25°C. Cells were harvested the next day by centrifugation at 4°C for 20 min at 5,000  $\times g$  in a Beckman Coulter Avanti-J-20 XPI centrifuge fitted with a JLA 8.1000 rotor. The cell paste was resuspended in 40 ml of 50 mM glycine buffer (pH 9.5) containing 5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride. Cell slurry was passed twice through a French pressure cell at 1,250 kPa to ensure breakage. Cell extracts were centrifuged at 4°C for 1 h at 43,667  $\times g$  in a Beckman Coulter Avanti-J-25I centrifuge fitted with a JA 25.50 rotor. The supernatant was decanted and treated with a few crystals of DNase for 20 min at

\* Corresponding author. Mailing address: 144A Enzyme Institute, 1710 University Ave., Madison, WI 53726-4087. Phone: (608) 262-7379. Fax: (608) 265-7909. E-mail: escalante@bact.wisc.edu.

<sup>†</sup> Present address: Department of Microbiology University of Illinois-Urbana, Urbana, Ill.

TABLE 1. Strains and plasmids

Strain or plasmid <sup>a</sup>	Genotype	Source or reference <sup>b</sup>
<b>Strains</b>		
<i>E. coli</i> JE3892	BL21(λDE3) F <sup>-</sup> <i>dcm ompT hsdS</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>gal</i> λ(DE3)	New England Biolabs
<i>S. enterica</i>		
TR6583	<i>metE205 ara-9 cobA</i> <sup>+</sup>	K. Sanderson via J. Roth
JE1293*	<i>cobA366::Tn10d(cat</i> <sup>+</sup> <i>)</i>	
JE7180*	<i>cobA366::Tn10d(cat</i> <sup>+</sup> <i>) eut1141 (ΔeutT)</i>	
JE7954*	<i>cobA366::Tn10d(cat</i> <sup>+</sup> <i>) eut1141 (ΔeutT)/pMmaCOBA4</i>	This study
JE7955*	<i>cobA366::Tn10d(cat</i> <sup>+</sup> <i>) eut1141 (ΔeutT)/pT7-7</i>	This study
<b>Plasmids</b>		
pMmaCOBA4	<i>bla</i> <sup>+</sup> <i>M. mazei</i> strain Gö1 P <sub>T7</sub> <i>cobA<sub>Mm</sub></i>	This study
pT7-7	<i>bla</i> <sup>+</sup>	42

<sup>a</sup> \*, Derivative of strain TR6583.

<sup>b</sup> Unless otherwise indicated, all strains are part of the laboratory collection.

room temperature and dialyzed twice for 30 min against 750 ml of 50 mM glycine buffer (pH 9.5) containing 5 mM DTT. After treatment, cell extracts were halved and purified independently.

Native CobA<sub>Mm</sub> protein was purified by using an ÄKTA Explorer fast-protein liquid chromatograph (Pharmacia). First, each half of the treated cell extract was loaded onto two 5-ml HiTrap Q Fast Flow columns (Amersham Biosciences) connected in a series. After a 20-ml wash with buffer A1 (50 mM glycine [pH 9.5], 5 mM DTT), CobA<sub>Mm</sub> protein was eluted in a linear gradient to 30% buffer B1 (50 mM glycine [pH 9.5], 1 M NaCl, 5 mM DTT) over 200 ml (0 to 300 mM NaCl). Fractions containing CobA<sub>Mm</sub> were pooled and dialyzed overnight against 50 mM glycine (pH 9.5), 4 M NaCl, and 5 mM DTT. After dialysis, each sample was applied to two 5-ml HiTrap Phenyl High Performance columns (Amersham Biosciences) in a series. After a 20-ml wash with buffer A2 (50 mM glycine [pH 9.5], 4 M NaCl, 5 mM DTT), CobA<sub>Mm</sub> was eluted with a linear gradient to 100% buffer B2 (50 mM glycine [pH 9.5], 4 M ethylene glycol, 5 mM DTT) over 100 ml. Fractions containing CobA<sub>Mm</sub> were pooled and dialyzed against buffer A1. The sample was concentrated to 10 ml by using an Amicon Centricon with a YM10 membrane (cutoff = 10 kDa). Each sample was purified by using an 8-ml Source 15Q column (Amersham Biosciences) After a 16-ml wash with buffer A1, CobA<sub>Mm</sub> was eluted with a linear gradient to 15% B1 over 80 ml (0 to 150 mM NaCl). The purity of CobA<sub>Mm</sub> protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (23), followed by Coomassie blue staining (32). Fractions enriched for CobA<sub>Mm</sub> protein were concentrated by using an Amicon Centricon device with a YM10 membrane and dialyzed against 10 mM glycine buffer (pH 9.5). Glycerol was added to dialyzed CobA<sub>Mm</sub> protein to a final concentration of 10% (vol/vol), and samples were stored at -80°C until used.

**Adenosyltransferase assays.** Assays were performed in sealed quartz cuvettes flushed with oxygen-free N<sub>2</sub>. Reactions contained 0.2 M Tris-Cl buffer (pH 8, 37°C), 50 μM HOCbl, 500 μM ATP, 500 μM CaCl<sub>2</sub>, 2.5 mM Ti(III)citrate reductant (26), and 2.5 μM CobA<sub>Mm</sub> protein. The reaction rate was monitored by determining the decrease in absorbance at 388 nm (9, 20). For flavodoxin-dependent assays, Ti(III)citrate was replaced by FldAH<sub>6</sub> protein (C terminus H<sub>6</sub>-tagged flavodoxin, 2 μM), Fpr protein [ferredoxin(flavodoxin):NADPH oxidoreductase, 2 μM], and NADPH (500 μM) (16). The amount of adenosylcobalamin (AdoCbl) synthesized after 30 min was monitored by determining the decrease in absorbance at 525 nm after photolysis for 10 min on ice as described previously (41).

**<sup>31</sup>P-NMR spectroscopy.** A 10-ml corrinoid adenosylation reaction mixture containing Ti<sup>3+</sup> citrate (3 mM), ATP (100 μM), HOCbl (100 μM), CaCl<sub>2</sub> (500 μM), and CobA<sub>Mm</sub> protein (5 μM) in 0.2 M Tris-Cl buffer (pH 8, 37°C) was incubated for 2 h at 37°C. After incubation, EGTA was added to 20 mM, reactions were concentrated in a SpeedVac overnight at room temperature, and 100% D<sub>2</sub>O was added to 6%. Orthophosphate, pyrophosphate, and triphosphate standards were added to 100 μM when indicated. The data were collected at the regional nuclear magnetic resonance (NMR) facility at Madison at the University of Wisconsin-Madison.

## RESULTS

**The protein encoded by *M. mazei* Gö1 open reading frame (ORF) MM3138 functions as an ATP:co(I)rrinoid adenosyl-**

**transferase in vivo.** The predicted amino acid sequence of the *M. mazei* Gö1 MM3138 protein (referred to herein as CobA<sub>Mm</sub>) was 31% identical to the *S. enterica* CobA protein (Fig. 1). This suggested that the *MmCobA* amino acid sequence could be the ATP:co(I)rrinoid adenosyltransferase of this methanogenic archaeon. The presence of a CobA-type, short ATP-binding P-loop motif (2) (Fig. 1) strengthened this hypothesis. Noteworthy differences between the primary amino acid sequences of these proteins were the absence in CobA<sub>Mm</sub> of residues 4 to 25 of CobA<sub>Se</sub>. A deletion of these residues in CobA<sub>Se</sub> severely impairs the interactions between CobA<sub>Se</sub> and flavodoxin A (8). Three other shorter stretches of amino acids in CobA<sub>Se</sub> were missing in CobA<sub>Mm</sub>, while a 15-residue stretch present in CobA<sub>Mm</sub> was absent in CobA<sub>Se</sub> (Fig. 1). We speculate that, based on these differences, the three-dimensional structures of these proteins may differ significantly. Efforts to solve the three-dimensional crystal structure of CobA<sub>Mm</sub> are under way.

To demonstrate the function of CobA<sub>Mm</sub> in vivo, ORF MM3138 was amplified from *M. mazei* strain Gö1 DNA, and the amplification product was cloned into overexpression vector pT7-7 (42); the resulting plasmid was named pMmaCOBA4 (Table 1). Plasmid pMmaCOBA4 was transformed into *S. enterica metE cobA eutT* strain JE7180, and the ability of the resulting strain (JE7954) to grow under conditions that required low or high levels of AdoCbl was assessed. A control strain was constructed by transforming the cloning vector pT7-7 into strain JE7180, yielding strain JE7955.

**Low Cbl requirement test.** Low Cbl levels (1 nM) satisfy the methionine requirement of an *S. enterica metE* strain during growth on glucose or glycerol (45, 49). Strains lacking Cbl-independent methionine synthase (MetE) methyltransferase activity methylate homocysteine via the Cbl-dependent methionine synthase (MetH) enzyme (18). During aerobic growth on glycerol, a culture of strain JE7954 (*metE cobA eutT/pMmacobA*<sup>+</sup>) reached the same cell density as a culture of strain TR6583 (*metE cobA*<sup>+</sup> *eutT*<sup>+</sup>) (Fig. 2A, solid symbols), but the rate of growth of strain TR6583 (*cobA*<sup>+</sup>) was faster by a factor of 2. The positive effect of CobA<sub>Mm</sub> on the conversion of cobinamide to AdoCbl was substantial compared to the growth behavior of control strains (Fig. 2A, open symbols).

**High Cbl requirement test.** To grow on ethanolamine as a sole carbon and energy source, *S. enterica* must synthesize AdoCbl before the EutR regulatory protein (29) can activate



FIG. 1. Alignment of CobA from *S. enterica* with archaeal homologs. The primary amino acid sequence of CobA<sub>Se</sub> is 31% identical and 52% similar to that of CobA<sub>Mm</sub>. The dark-shaded box indicates the P-loop ATP-binding motif, while the light-gray box indicates regions of conservation. Asterisks denote absolutely conserved amino acid residues. Sen, *Salmonella enterica*; Mma, *Methanosarcina maei* strain G61; Mac, *Methanosarcina acetivorans* strain C2A; Hsa, *Halobacterium salinarum* strain NRC-1; Pae, *Pyrobaculum aerophilum*; Pab, *Pyrococcus abyssi*; Pfu, *Pyrococcus furiosus*; Pho, *Pyrococcus horikoshii*; Tac, *Thermoplasma acidophilum*; Tvo, *Thermoplasma volcanium*. Alignment was generated by using DNA\* software package v.1.66 (DNASTAR, Inc.) without adjustments.

transcription of the ethanolamine utilization (*eut*) operon (9, 34) that encodes functions needed to catabolize ethanolamine (6, 7, 21, 30, 31, 36, 37). Growth of *S. enterica* strain JE7954 [*cobA eut* (*eutT*)/pMmaCOBA4 *cobA*<sup>+</sup><sub>Mm</sub>] on ethanolamine requires high levels of AdoCbl (≥150 nM [6]). As shown in Fig. 2B, the CobA<sub>Mm</sub> enzyme did not compensate for the lack

of CobA<sub>Se</sub> enzyme during growth of strain JE7954 on ethanolamine as sole carbon and energy source. There are several possible explanations for why the CobA<sub>Mm</sub> enzyme did not support growth on ethanolamine as efficiently as the CobA<sub>Se</sub> enzyme did. For example, CobA<sub>Mm</sub> may not have been expressed at a high enough level to support wild-type growth, it

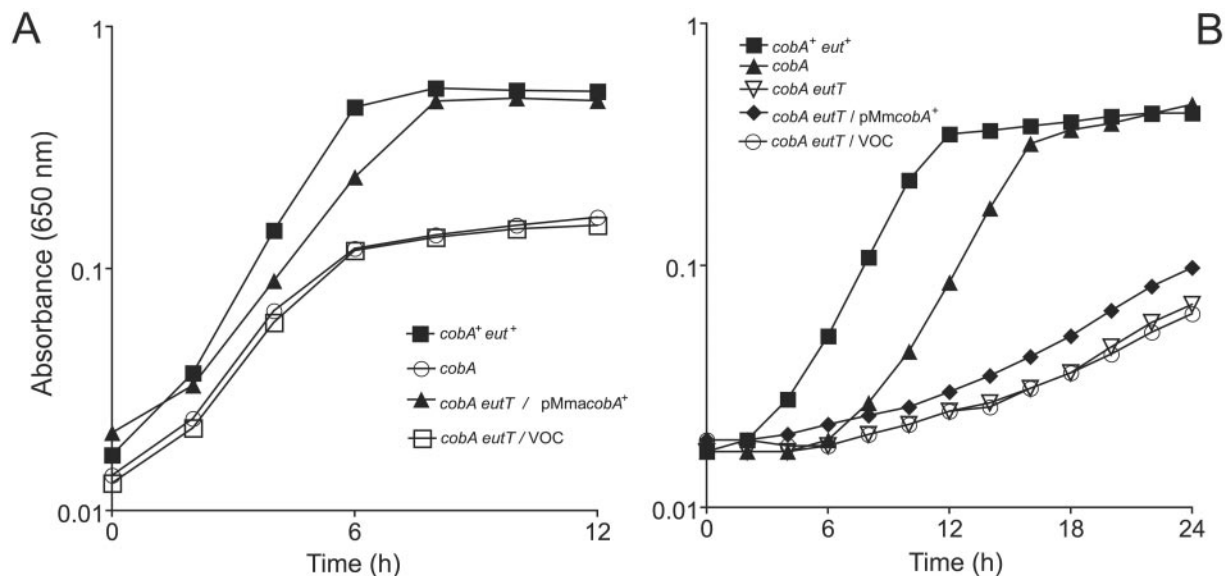


FIG. 2. *cobA*<sup>+</sup><sub>Mm</sub> compensates for the lack of *cobA*<sub>Se</sub> under conditions that require low levels of AdoCbl for growth. (A) Growth behavior of *S. enterica* strains grown in minimal medium containing glycerol as sole carbon and energy source. (B) Growth in minimal medium containing ethanolamine as sole carbon and energy source. Derivatives of strain JE7180 [*cobA366::Tn10d(cat<sup>+</sup>) eut1141 (Δ*eutT*)*] carrying plasmids pMmaCOBA4 *cobA*<sup>+</sup><sub>Mm</sub> (strain JE7954) or pT7-7 (VOC, strain JE7955) were used to investigate CobA<sub>Mm</sub> function in vivo. Strains TR6583 (*cobA*<sup>+</sup><sub>Se</sub>) and JE1293 [*cobA366::Tn10d(cat<sup>+</sup>)*] were used as controls. VOC, vector-only control.

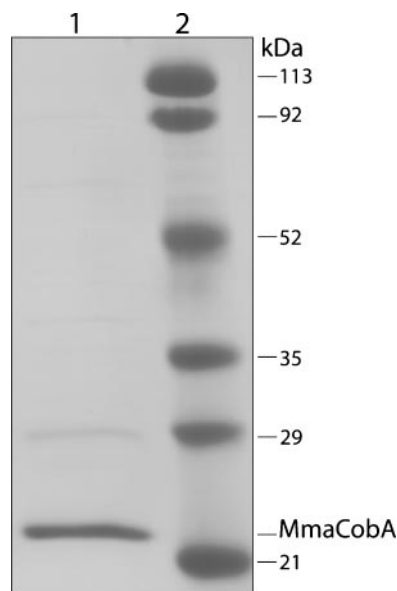


FIG. 3. CobA<sub>Mm</sub> protein purity. CobA<sub>Mm</sub> protein was isolated as described in Materials and Methods. After Coomassie blue staining, protein purity was quantified by using a Fotodyne.

may not have been stably maintained, or its interactions with flavodoxin may have been suboptimal. At present, no data favoring any of these possibilities is available, however. Nevertheless, the data presented in Fig. 2A support the conclusion that the CobA<sub>Mm</sub> protein has activity that can attach the adenosyl upper ligand to the corrin ring of cobinamide in vivo, allowing its conversion to AdoCbl and the use of the latter in methionine synthesis.

**Initial biochemical characterization of CobA<sub>Mm</sub>.** CobA<sub>Mm</sub> enzyme was purified from *E. coli*. We obtained 8 mg of CobA<sub>Mm</sub> protein per liter of culture grown under the conditions described above; CobA<sub>Mm</sub> was calculated to be 88% homogeneous (Fig. 3). In vitro corrinoid adenosyltransferase assays with CobA<sub>Mm</sub> protein were performed by using published protocols for assaying CobA<sub>Se</sub> activity (9).

**Reductant and pH<sub>opt</sub>.** Potassium borohydride (KBH<sub>4</sub>) was tested as a reducing agent in lieu of Ti<sup>3+</sup> citrate but, for unknown reasons, it was detrimental to the activity of the enzyme. CobA<sub>Mm</sub> activity was assayed at various pH values. Under conditions where Mg<sup>2+</sup> ion concentration was saturating for CobA<sub>Se</sub>, the highest rate of AdoCbl synthesis occurred at pH 8 (e.g., at pH 7.5 the specific activity is 0.75, at pH 8 the specific activity is 1.02, and at pH 8.5 specific activity is 0.94 nmol min<sup>-1</sup> mg<sup>-1</sup>).

**Cation requirement.** We tested the effect of metal ions on CobA<sub>Mm</sub> activity under conditions that used Ti<sup>3+</sup> citrate as a reductant at pH 7. Ca<sup>2+</sup> ions stimulated CobA<sub>Mm</sub> activity 2.5-fold over activities measured in reaction mixtures where metal salts were not added. Co<sup>2+</sup>, Ni<sup>2+</sup>, and Zn<sup>2+</sup> ions stimulated the CobA<sub>Mm</sub> activity 1.75-, 1.5-, and 1.25-fold, respectively. Surprisingly, and unlike CobA<sub>Se</sub>, CobA<sub>Mm</sub> activity was substantially inhibited by Mn<sup>2+</sup> ions (60% reduction) relative to the no-additions control, and Mg<sup>2+</sup> ions did not significantly improve enzyme activity (Fig. 4). In light of the above results, Ca<sup>2+</sup> ions were present at 0.5 mM in all subsequent assays.

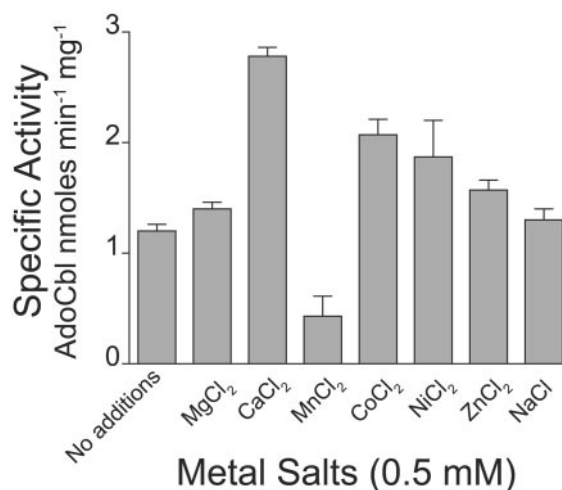


FIG. 4. CobA<sub>Mm</sub> enzyme activity is enhanced by the addition of Ca<sup>2+</sup> ions. Various metal salts were tested for their effect on CobA<sub>Mm</sub> activity in vitro. Salts were added to the reaction mixture containing 0.2 M Tris-Cl buffer (pH 8, 37°C), CobA<sub>Mm</sub> (2 μM), ATP (100 mM), HOCbl (50 μM), and Ti(III) citrate (2.5 mM) as a reductant.

**Kinetic parameters and corrinoid substrate preference.** We used the optimized conditions described above to gain insights into the affinity of CobA<sub>Mm</sub> for its substrates and the velocity of the reaction. From the data shown in Fig. 5 we calculated a  $K_m^{ATP}$  of 3 μM when Cbl was held at 0.05 mM. When the ATP was held at 0.5 mM and the concentration of Cbl was varied, we calculated a  $K_m^{Cbl}$  of 0.9 μM and a  $V_{max}$  of 3 nmol min<sup>-1</sup>.

While the kinetic analyses were performed with HOCbl as substrate, the uncertain physiological role of CobA<sub>Mm</sub> in vivo prompted us to test the ability of the enzyme to use Cbi as substrate since it lacks the lower 5,6-dimethylbenzimidazole ligand. Under substrate saturation conditions, the specific activities when Cbl or Cbi was used as a substrate was 1.56 or 0.90 nmol min<sup>-1</sup> mg<sup>-1</sup>, respectively. Therefore, under the conditions of the in vitro assay, CobA<sub>Mm</sub> preferred Cbl over Cbi as a substrate.

**Nucleoside triphosphate substrate.** The activity of CobA<sub>Mm</sub> as a function of different nucleoside triphosphates was determined. The data in Fig. 6A were plotted relative to the activity of the enzyme when ATP was the substrate. Unlike CobA<sub>Se</sub>, the base of nucleoside substrates tested had a significant negative effect on CobA<sub>Mm</sub> activity. The most dramatic effect was obtained with CTP. Although CTP is a better substrate than ATP for CobA<sub>Se</sub> (15), it was a poor substrate for CobA<sub>Mm</sub>. A second difference between CobA<sub>Se</sub> and CobA<sub>Mm</sub> was observed when deoxynucleoside triphosphates (dNTPs) were used. dNTPs are not substrates for CobA<sub>Se</sub> (15), but CobA<sub>Mm</sub> used dNTPs as substrates, and its activity with dCTP was equivalent to the activity measured with ATP. The most striking difference between CobA<sub>Se</sub> and CobA<sub>Mm</sub> was observed with ADP. Although ADP is not a substrate for CobA<sub>Se</sub> (15), CobA<sub>Mm</sub> retained 37% of its activity when ADP substituted for ATP. This result suggested that the γ phosphate was likely the primary phosphate for ATP coordination.

**Triphosphate is the by-product of the CobA<sub>Mm</sub> reaction.** In an effort to identify the phosphate by-product of the CobA<sub>Mm</sub> reaction, we tested the sensitivity of the enzyme to

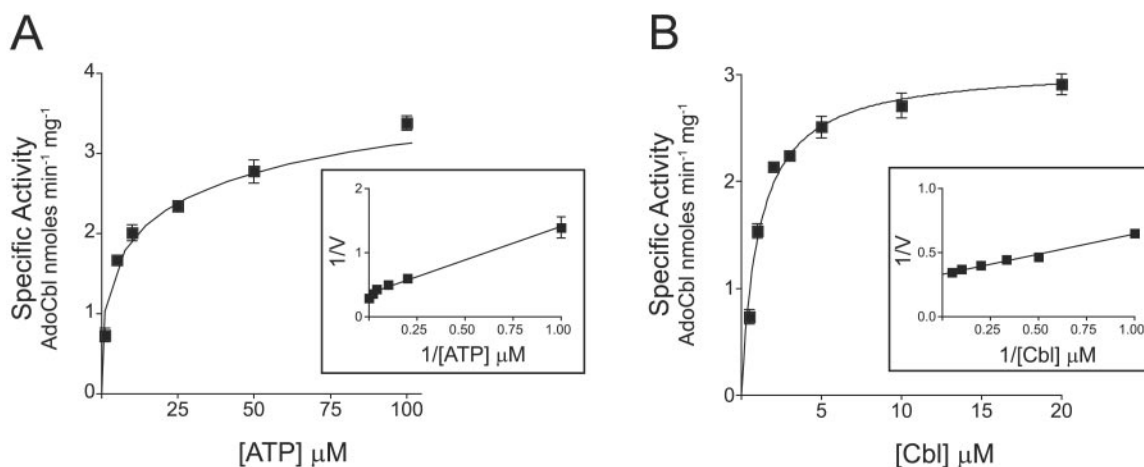


FIG. 5. Kinetic analysis of the CobA<sub>Mm</sub>-catalyzed reaction. (A) HOCbl was held constant at 0.05 mM, while the concentration of ATP was varied.  $V_{\max}$  was determined to be 3 nmol of AdoCbl formed min<sup>-1</sup> mg<sup>-1</sup>; the apparent  $K_m^{\text{ATP}}$  was calculated at 3 μM. (B) The ATP concentration was held constant at 0.50 mM, while the HOCbl concentration was varied.  $V_{\max}$  was 3 nmol of AdoCbl formed min<sup>-1</sup> mg<sup>-1</sup>, and the apparent  $K_m^{\text{HOCbl}}$  was calculated to be 1 μM.

triphosphate (PPP<sub>i</sub>), pyrophosphate (PP<sub>i</sub>), or orthophosphate (P<sub>i</sub>) (all at 1 mM, Fig. 6B). CobA<sub>Mm</sub> was not inhibited by the presence of PPP<sub>i</sub> and in fact was slightly stimulated (Fig. 6B). This stimulatory effect was not due to a simple increase in ionic strength; the addition of up to 10 mM NaCl to the assays had no effect on the rate of catalysis (data not shown). PP<sub>i</sub> was a weak inhibitor (28% decrease in specific activity), and P<sub>i</sub> had no significant effect. CobA<sub>Sc</sub> is strongly inhibited by PPP<sub>i</sub> and has been shown to release PPP<sub>i</sub> as a reaction by-product (15). In light of this information, we hypothesized that CobA<sub>Mm</sub> probably cleaved PPP<sub>i</sub> to PP<sub>i</sub> and P<sub>i</sub> as part of the catalytic cycle. We addressed this possibility using <sup>31</sup>P-NMR spectroscopy (Fig. 7). The <sup>31</sup>P-NMR spectrum showed an accumulation of PPP<sub>i</sub> in the reaction mixture, no increase in P<sub>i</sub> signal, and no detectable PP<sub>i</sub> peak. In Fig. 7A, the signal proximal to Cbl was not identified. We

speculate that it was likely the result of ion coordination by the phosphate moiety in the nucleotide loop of Cbl. In Fig. 7C and D, the doublet proximal to the α-PPP<sub>i</sub> signal was not identified and may arise from ion interactions with PPP<sub>i</sub>. On the basis of this information we concluded that CobA<sub>Mm</sub> does not cleave PPP<sub>i</sub> to PP<sub>i</sub> and P<sub>i</sub>. The absence of inhibition by PPP<sub>i</sub> and only weak inhibition by PP<sub>i</sub> may reflect the importance of purine ring coordination in substrate binding to the enzyme active site.

## DISCUSSION

We presented here experimental evidence supporting the annotation of an archaeal gene encoding a protein with CobA-type ATP:co(I)rrinoid adenosyltransferase activity. To reflect these findings, we propose that ORF MM3138 from the methanogenic

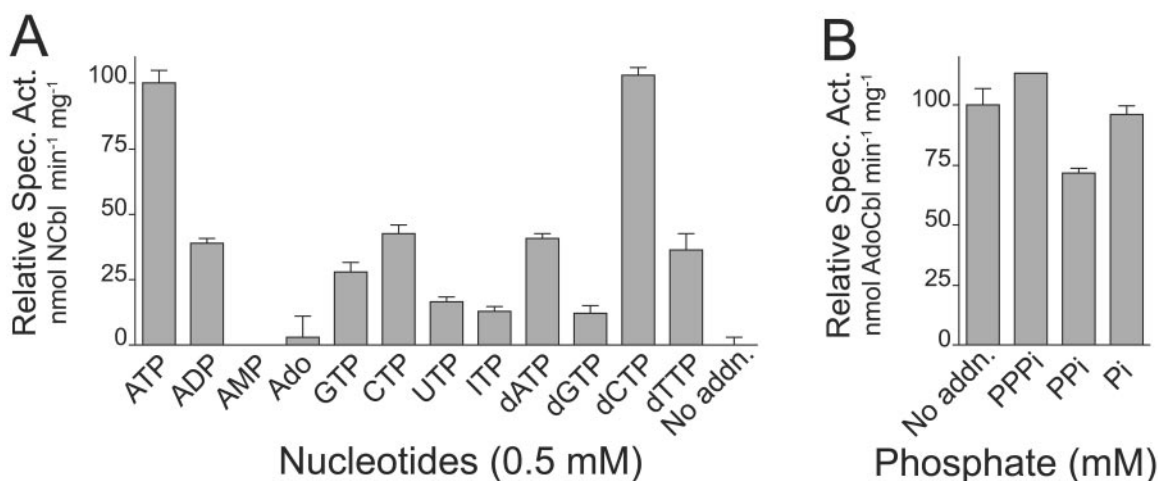


FIG. 6. CobA<sub>Mm</sub> protein interacts with the 2'-OH and the γ phosphate of ATP but is not inhibited by PPP<sub>i</sub>. (A) Specificity of the CobA<sub>Mm</sub> enzyme for its nucleoside triphosphate substrate. When added, ADP was present in the reaction mixture at 500 μM. The reaction rate for a mixture containing ATP was 2.3 nmol of AdoCbl min<sup>-1</sup> ± 0.1. Ado, adenosine. (B) When added to the reaction mixture, PPP<sub>i</sub>, PP<sub>i</sub>, or P<sub>i</sub> was present at 1 mM. The reaction rate for a mixture with no additions was 1.8 nmol of AdoCbl min<sup>-1</sup>.

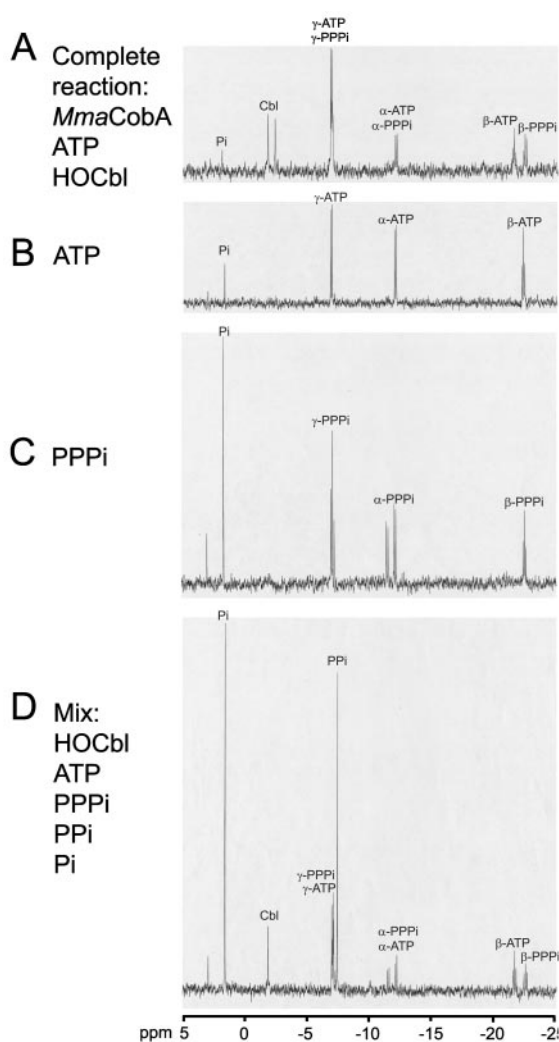


FIG. 7.  $^{31}\text{P}$ -NMR analysis of  $\text{CobA}_{Mm}$  reaction by-products. Strong signals for the  $\beta$  phosphate of ATP (centered at  $-21.51$  ppm) and  $\text{PPPi}$  (centered at  $-22.39$  ppm) were observed after incubation of  $\text{CobA}_{Mm}$  with its substrates. No signal was detected from  $\text{PPi}$  at  $-7.41$  ppm. (A) Complete reaction mixture; (B) ATP standard; (C)  $\text{PPPi}$  standard; (D) mixture of ATP,  $\text{PPPi}$ ,  $\text{PPi}$ ,  $\text{P}_i$ , and  $\text{HOcbl}$ .

archaeon *Methanosarcina mazei* strain Gö1 be hereafter referred to as *cobA*.

**Potential differences between archaeal and bacterial  $\text{CobA}$  enzymes.** Although  $\text{CobA}_{Mm}$  and  $\text{CobA}_{Se}$  may have evolved from a common ancestor (Fig. 1), there could be significant differences between the mechanisms of catalysis used by these enzymes. Proper positioning of the ATP substrate in the active site of  $\text{CobA}_{Se}$  is critical to catalysis. However, biochemical data suggest that  $\text{CobA}_{Se}$  and  $\text{CobA}_{Mm}$  may not bind ATP the same way. For example, the absolute requirement of  $\text{CobA}_{Se}$  for the 2'-OH of the ribose in ATP is not shared by  $\text{CobA}_{Mm}$ , suggesting that  $\text{CobA}_{Mm}$  presents the target (5'-C) for the nucleophilic attack by  $\text{Co(I)}$  by different means. The role of the base of NTPs is also significantly different between the archaeal and bacterial enzymes. Although  $\text{CobA}_{Mm}$  uses ITP, GTP, CTP, and UTP as substrates,  $\text{CobA}_{Se}$  can use them more efficiently. In fact,  $\text{CobA}_{Se}$  prefers CTP and UTP over ATP

(15). It is therefore likely that  $\text{CobA}_{Mm}$  interacts with the purine ring more than  $\text{CobA}_{Se}$ , which only forms a single H-bond between the carbonyl oxygen of Asn37 and the amino group of adenine (2). Therefore, in  $\text{CobA}_{Mm}$ , positioning of the 5'-C may be mediated by interactions with the  $\gamma$  phosphate and the purine ring rather than by interactions with the  $\alpha$  and  $\beta$  phosphates and the 2'-OH as in  $\text{CobA}_{Se}$ . This difference in ATP binding was unexpected considering the relatedness of the two enzymes.

$\text{CobA}_{Se}$  and  $\text{CobA}_{Mm}$  may interact differently with its corrinoid substrate. Many of these differences may be due to the absence of an N-terminal helix in  $\text{CobA}_{Mm}$ . Residues 1 to 23 of  $\text{CobA}_{Se}$  form an N-terminal  $\alpha$  helix that closes over the occupied active site (2).  $\text{CobA}_{Mm}$  has only 10 residues before the ATP-binding P-loop motif (Fig. 1). A plausible explanation would be that the higher apparent  $K_m$  of  $\text{CobA}_{Mm}$  for Cbl and the sluggishness of the enzyme may be attributable to the absence of the N-terminal helix. It is important to note, however, that an N-terminal truncation of  $\text{CobA}_{Se}$  results in a more active enzyme, as long as the substrates are present at saturating levels (8), suggesting that an enzyme lacking the N-terminal helix undergoes faster substrate binding and product release. The  $V_{max}$  for  $\text{CobA}_{Mm}$  is also ca. 17% of that of  $\text{CobA}_{Se}$  (3 versus 18 nmol of  $\text{AdoCbl min}^{-1} \text{mg}^{-1}$ ). This decreased  $\text{CobA}_{Mm}$  activity may be a consequence of the overexpression/purification procedures or suboptimal in vitro assay conditions, or it may represent a true difference in enzyme catalysis. Additional work is required to determine the reasons for this important difference.

The marked difference in the ability between  $\text{CobA}_{Mm}$  and  $\text{CobA}_{Se}$  in the use ADP as a substrate suggests significant variations in how these enzymes bind ATP. The three-dimensional structure of  $\text{CobA}_{Se}$  complexed with  $\text{MgATP}$  and Cbl shows the  $\text{Mg}^{2+}$  ion octahedrally coordinated by two nonbridging phosphate oxygens from the  $\alpha$ - and  $\beta$ -phosphates, the hydroxyl group of Thr42, a carboxylate oxygen of Glu128 and two water molecules. This coordination is similar to that observed in other enzymes (e.g.,  $\text{F}_1\text{ATPase}$ ), with some differences. The three-dimensional structure of  $\text{CobA}_{Mm}$  complexed with substrates is needed to better understand how  $\text{CobA}_{Mm}$  binds ATP, its cation, and its corrinoid substrate. Efforts to obtain a three-dimensional structure of  $\text{CobA}_{Mm}$  are currently under way.

**$\text{CobA}_{Mm}$  may be a  $\text{Ca}^{2+}$ -dependent ATP:co(I)rrinoid adenosyltransferase.** ATP-binding enzymes use divalent cations to coordinate the triphosphate and partially shield the negative charge. The same is true for cobalamin adenosyltransferases. It is known that  $\text{CobA}_{Se}$  prefers  $\text{Mn}^{2+}$  but will use  $\text{Mg}^{2+}$  ions in vitro (40). A second class of ATP:co(I)balamin adenosyltransferase found in *S. enterica* is encoded by the *pduO* gene of the 1,2-propanediol utilization (*pdu*) operon. The PduO enzyme prefers  $\text{Mg}^{2+}$  as a cation (19, 20). We were very surprised to see that  $\text{Ca}^{2+}$  ions stimulated activity 2.5-fold, but  $\text{Mg}^{2+}$  ions did not stimulate activity, and  $\text{Mn}^{2+}$  ions inhibited the enzyme (Fig. 4). In these assays,  $\text{CobA}_{Mm}$  was not treated with chelating agents, so the presence of any accessory bound cations cannot be ruled out. However, the 2.5-fold stimulation of enzyme activity was specific for  $\text{Ca}^{2+}$ . There are only a few examples in the literature of  $\text{CaATP}$  binding enzymes, e.g., the human heat shock protein 70 (hHsp70) (27, 35),  $\beta$ -actin (10), and synapsin I (14, 46). It is important to note that hHsp70 and

$\beta$ -actin have been crystallized complexed with MgATP and seem to require a divalent cation for ATP binding, whereas synapsin I specifically requires CaATP for enzyme activity (5, 14). Although it is possible that CobA<sub>Mm</sub> requires Ca<sup>2+</sup> ions for structural integrity and not ATP binding, a possibility exists that CobA<sub>Mm</sub> may be a unique CaATP:co(I)rrinoid adenosyltransferase.

**Can *Salmonella* FldA serve as the electron shuttle protein for CobA<sub>Mm</sub>?** In *S. enterica*, the physiological electron transfer protein for the CobA enzyme is flavodoxin (FldA) (16). Although the *Methanosarcina* genomes contain flavodoxin homologs, corrinoid adenosyltransferases in these organisms may use one of several ferredoxins or other unknown means to generate co(I)rrinoid substrates for CobA<sub>Mm</sub>. The inability of CobA<sub>Mm</sub> to support the growth of *S. enterica* on ethanolamine may be a reflection of poor coupling between *S. enterica* FldA and CobA<sub>Mm</sub> proteins. If SenFldA is indeed the reductant for CobA<sub>Mm</sub>, their coupling may be insufficient to support growth on ethanolamine. However, even if poor, such coupling satisfies the Cbl requirement of the cell for methionine synthesis (Fig. 2A). In vitro assays using possible reductants from *M. mazei* are needed to address the question of co(II)rrin reduction in archaea.

**Why does *Methanosarcina* need an adenosyltransferase?** Methanogenic archaea use pseudo-B<sub>12</sub>, 5-hydroxybenzimidazolylcobamide, or cobalamin as a methyl donor (11, 12, 22, 33, 38), and sequenced genomes contain homologs of B<sub>12</sub>-dependent ribonucleotide reductases (43). At present, it is unclear what role the CobA<sub>Mm</sub> enzyme plays in vivo. It is possible that, like CobA<sub>Se</sub>, CobA<sub>Mm</sub> is needed to attach the adenosyl upper ligand to an intermediate of the corrin ring biosynthetic branch of the pathway, to adenosylate corrinoids from its environment that may or may not contain a lower ligand, or both (47, 50). Very little is known about B<sub>12</sub>-dependent metabolism in archaea. Biochemical data suggest salvaging of incomplete corrinoids and/or de novo biosynthesis proceeds through an adenosylated intermediate (47). However, details of the de novo biosynthetic pathway, transport, and precursor salvaging in archaea remain to be elucidated.

#### ACKNOWLEDGMENTS

This study was supported in part by NIH grant GM40313 to J.C.E.-S. N.R.B. was supported in part by a Howard Hughes Predoctoral Fellowship.

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